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Original Article

Glycyrrhizin attenuates tissue injury and reduces neutrophil accumulation in experimental acute pancreatitis

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Abstract: Leukocyte infiltration and acinar cell injury are characteristic features of acute pancreatitis (AP). However, the signaling pathways regulating inflammation and accumulation of leukocytes into pancreas tissue remains poorly elucidated. In the current study, we investigated the effects of Glycyrrhizin (GZ) on cerulein-induced AP in mice. AP was induced in male C57BL/6 by intraperitoneal injection of 50 μg/kg cerulein hourly, with a total of 7 times. 1 hour after the last injection of cerulean, mice were treated with either 35 or 70 mg/kg of GZ. Serum amylases and lipases were measured using automated chromogenic assay, MCP-1 and MIP-2 concentrations were measured in the serum by ELISA, and the number of infiltrated inflammatory cells in the pancreas were evaluated by flow cytometry. We found that GZ treatment resulted in reduction (i) both amylase and lipase activities, (ii) the serum levels of both MCP-1 and MIP-2; and (iii) markedly attenuated cerulein-induced histopathological alternations and water contents. Furthermore, we observed that GZ significantly decreased the number of infiltrated monocytes and neutrophils into the pancreas tissue. In conclusion, we demonstrate that GZ attenuates AP signs and inhibits inflammatory cell recruitments into pancreas.

Keywords: Glycyrrhizin, tissue injury, neutrophil accumulation, leukocyte infiltration, experimental acute pancreatitis

Introduction

Acute pancreatitis (AP) is considered as an inflammatory disease with a high mortality and morbidity. Although the mortality rate of AP has been decreased over the past decades, only a few treatment options are currently available [1]. Uncontrolled activation of pancreatic enzymes and migration of inflammatory cells particularly neutrophils are two man hallmarks of the pathogenesis of AP [2, 3]. Pro-inflammatory mediators and cytokines believed to be involved in pathophysiology of AP, as many of them such as tumor necrosis factor α (TNF-α), interleukins 6 (IL-6), IL-8 and monocyte chemoattractant protein-1 (MCP-1) have been shown elevated in the plasma of patients with AP [4-7]. Of interests, MCP-1 and IL-8 is increased in the early stage of AP, indicating that MCP-1 and IL-8 may participate as early triggers for inducing inflammatory cascades in AP and they could be use for as predictor biomarkers for diagnosis of AP [4, 8].

Mounting evidence indicate that migration and accumulation of leukocytes particularly neutrophils and subsequently their activation play a vital role in local inflammation and distance organ failure in AP [9]. Migration of inflammatory cells into the tissue during inflammation and tissue injury is highly regulated by inflammatory cytokines such as TNF-α, IL-6, MCP-1 and IL-8 as well as adhesion molecules. Moreover, leukocytes excessive have been suggested to be contributed in organ dysfunction in AP and leukocyte depletion reduces severity of pancreatitis and lung injury in animal models [10].

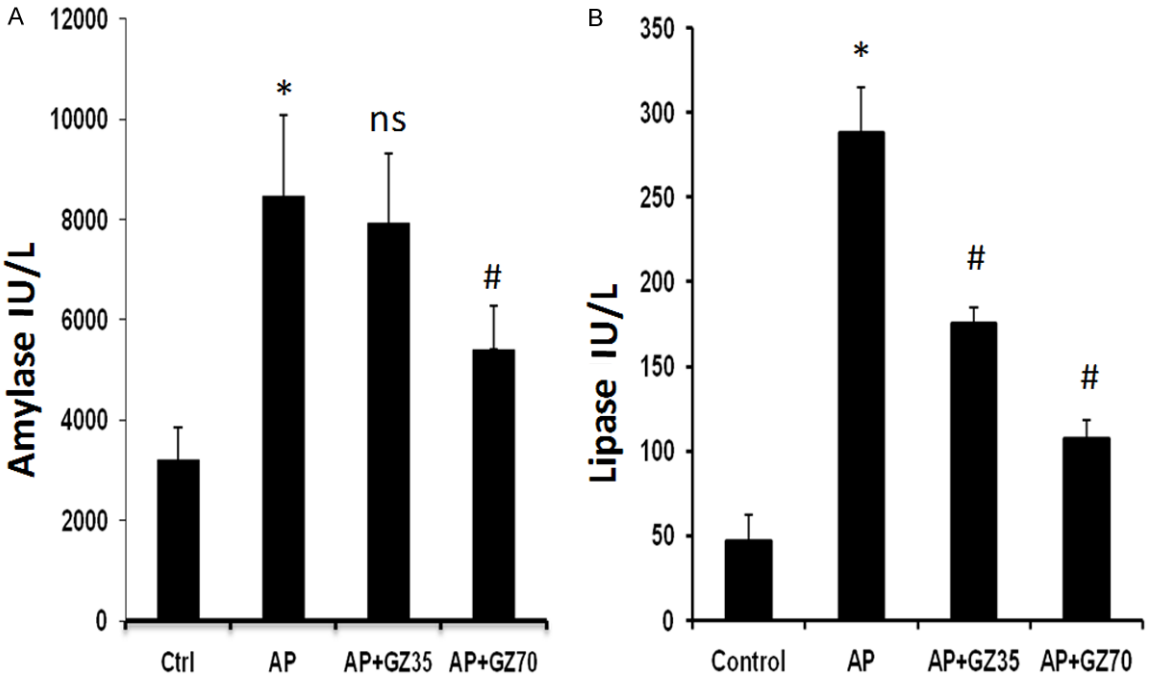

Figure 1. The effects of Glycyrrhizin on amylase and lipase activities. Blood samples were harvested from mice 12 hours after administration of Glycyrrhizin. The administration of GZ in mice significantly reduced the increase of amylase (A) and lipase (B) induced by cerulein. Each value is the mean ± SEM for 6-8 mice and significant levels were considered at $P<0.05$. *$P<0.05$ compared with the control mice; #$P<0.05$ compared with the AP group; ns; non-significant.

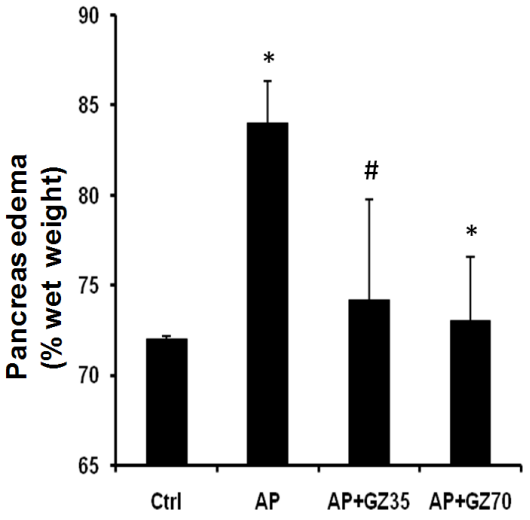

Figure 2. Effects of Glycyrrhizin on pancreas edema. The administration of Glycyrrhizin in mice significantly reduced the pancreas edema formation induced by cerulein. Each value is the mean ± SEM for 6-8 mice and significant levels were considered at $P<0.05$. *$P<0.05$ compared with the control mice; #$P<0.05$ compared with the AP group.

The root of Glycyrrhiza glabra (licorice) is widely used as a traditional medicine in Chinese and Persian herbal medicine [11] Glycyrrhizin (GZ) is one of derivatives of licorice and has been used as sweetener agent in Europe and Asia for many years. GZ have been shown to exhibit many pharmacological effects such as anti-inflammatory, anti-oxidative and hepatoprotective effects [12, 13]. For example, GZ have been used for more than 20 years in patients with chronic hepatitis in Japan [14]. It have been recently demonstrated that GZ treatment leads to attenuating of inflammation and tissue injury in animal models including spinal cord injury [15], experimental allergic asthma [16] and LPS-induced acute lung injury [17]. Nevertheless, the potential effect of GZ on AP has not yet been explored. In the current study, we evaluated the anti-inflammatory effects of GZ on cerulein-induced AP in mice and found that GZ effectively attenuated AP through decreasing of MCP-1 and MIP-2 and inhibition of inflammatory cells infiltration into pancreas.

Material and methods

Induction of AP

Healthy 8 week-old C57 mice weighing 25-30 g were used for the experiments. Mice were pur-

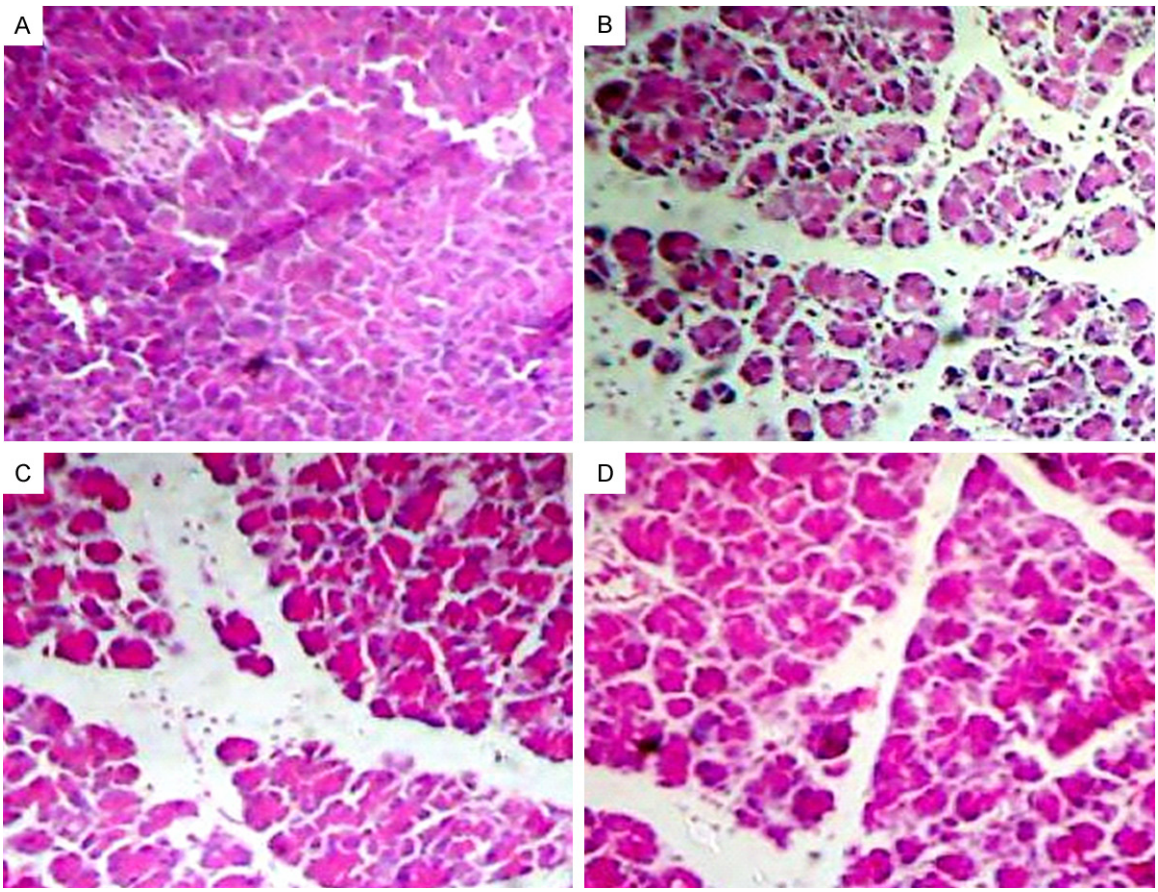

Figure 3. Effects of Glycyrrhizin on cerulein-induced histological alternations of pancreas. Representative hematoxylin/eosin stained sections of pancreas from a control mouse (A) demonstrates that the histological features of the pancreas were typical of a normal architecture. On the contrary, pancreas sections of cerulein-treated mice (B) demonstrate tissue damage characterized by interstitial edema, acinar cell necrosis, and inflammatory cell infiltrates. Pancreas section from mice that had received Glycyrrhizin at the dose of 35 (C) and 70 mg/kg (D) showed less histological alterations. The figure is representative of at least six experiments performed on different experimental days.

chased from Iran Pasture Institute (Tehran, Iran) and let them acclimate to the environment for at least 72 h after arrival in the laboratory. Experimental AP was induced as previously reported [18]. Briefly, Mice were randomly allocated into four groups (n = 8 for each group). The first group was treated hourly (× 7) with saline intraperitoneally and served as the control group. The second group was treated hourly (× 7) with cerulein (50 μg/kg, suspended in saline solution, i.p.) and served as the experimental AP group. In the AP + 35 GZ and AP + 70 GZ, mice got cerulein with the same procedure as that in the AP group, and 35 or 70 mg/kg of GZ (Sigma, Sigma Chemical, St. Louis, MO) were give intraperitoneally an hour after the last cerulein injection, respectively. Twelve hours later, mice were killed and blood samples were obtained by direct intracardiac puncture. Pancreas was removed immediately, divided in three equal parts. A part of it was used for flow cytometry analysis, the second part was used for determination of edema and the rest were fixed in formaldehyde for histopathological examinations.

Histopathological and biochemical examinations

Paraffin-embedded pancreas samples were sectioned (5 μm) and stained with hematoxylin/eosin. Pancreas sections were examined by an experienced pathologist (BN), who was not aware of the sample identity. Acinar cell injury/necrosis was quantified by morphometry as previously described [18]. For these studies, 10 randomly chosen microscopic fields were examined for each tissue sample, and the extent of

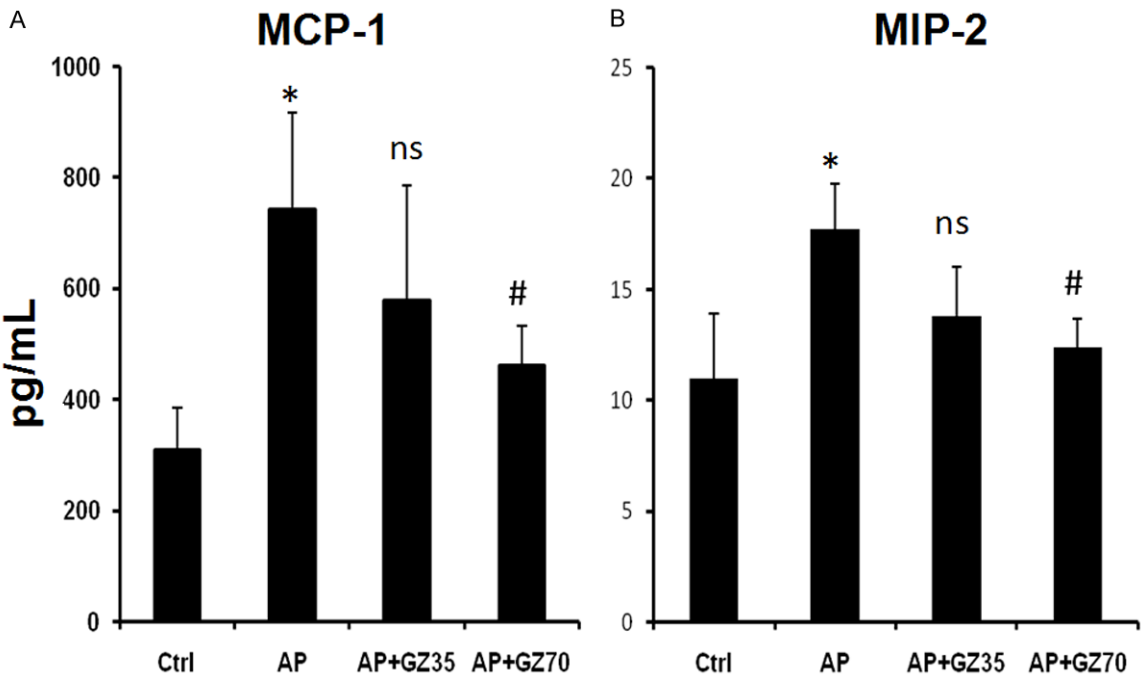

Figure 4. Effects of Glycyrrhizin on MCP-1 and MIP-2. Cytokines were measured by ELISA according to manufacturer's induction. Glycyrrhizin reduced serum levels of MCP-1 (A) and MIP-2 in a dose-dependent manner. Data are means ± SEM of 8 mice for each group and significant levels were considered at P<0.05. *P<0.05 compared with the control mice; #P<0.05 compared with the AP group; ns; non-significant.

acinar cell injury/necrosis and infiltration of inflammatory cells were evaluated. Amylase and lipase activities were detected in the serum of all experimental groups in a diagnostic Lab (Behsat Hospital, Sanadaj, Iran).

Determination of pancreas edema

The extent of pancreatic edema was assayed by measuring tissue water content as previously reported [18]. In brief, freshly obtained samples of pancreas from each group of mice were weighted on aluminium foil, dried for 12 h at 95°C, and reweighted. The difference between wet and dry tissue weight was calculated and expressed as a percent of tissue wet weight.

Isolation of pancreatic cells

Pancreatic cells were isolated from pancreas tissue as previously reported [19]. Briefly, pancreas gland was cut into small fragments and suspended in FACS buffer (Dulbecco's phosphate-buffer saline containing 10% fetal calf serum medium and 5 mM EDTA). Tissue fragments were more grinded by pipetting with 1ml sampler for 30 s. The remaining large fragments were allowed to sediment for 20 s, the supernatant collected and reserved. Then, the sedimented large fragments were again triturated and the total of collected supernatants from each pancreas was pooled, centrifuged (250 X g, 4°C, 25 min). The resulting cell pellets were then resuspended in FACS buffer for immediate staining for flow cytometry.

Flow cytometry analysis

Pancreatic cells were stained for CD45 antigen as previously reported [19] Isolated pancreatic cells from each mouse were washed three times with FACS buffer, resuspended in 200 µL of FACS buffer and then distributed equally into two 1.5 mL microtubes. In order to stain the cells for CD45 antigen, 10 µl rat anti-mouse CD45-PE/Cy5 (eBiosciences, San Diego, CA) was added to one tube and incubated for 45 min at 4°C in dark place. To determine cut-off values and true positive staining, 10 µl isotype control antibody conjugated with PE/Cy5 (eBioscience) was added to the second tube and incubated for 45 min at 4°C in dark place. Immunostained cells were washed three times, resuspended in FACS buffer and subjected to

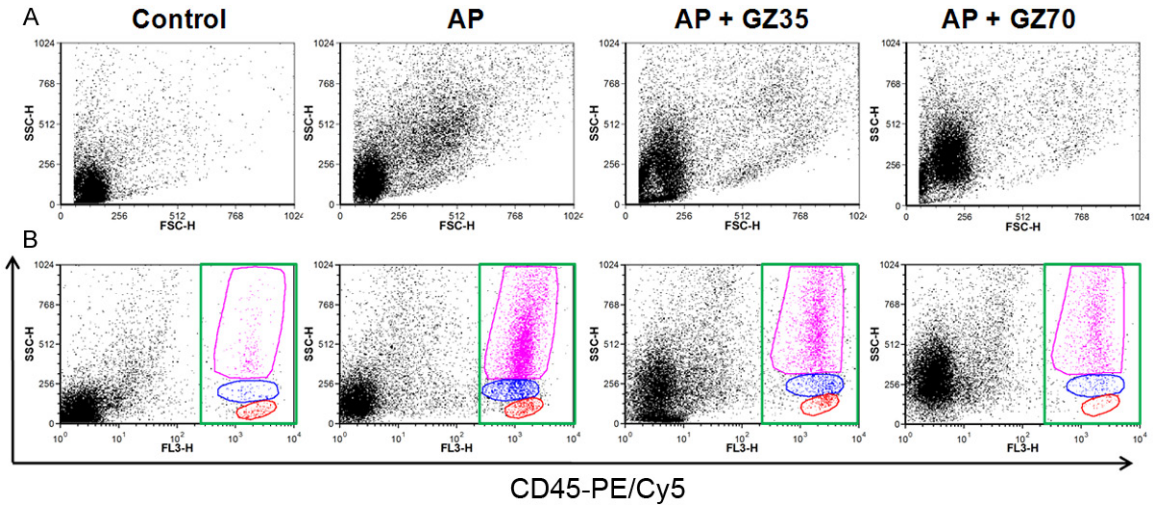

Figure 5. Flow cytometry analysis of inflammatory cells in pancreas of mice with AP. A: Representative forward and side scatter plot for isolated cell in the AP group. B: CD45/SSC gating was used to identify various leukocytes populations in the AP group. Green rectangle gate shows leukocytes population. Pink, blue and red gates show granulocytes, monocyte and lymphocyte population, respectively. Representative plots from 6-8 mice are shown.

flow cytometry using FACS Calibur (Beckman Dickinson, San Jose, CA) within an hour. FCAS data were analyzed by FCS Express software (De Novo Software, Los Angeles, CA).

Statistics

Numeral data are presented as mean ± SD from 8 mice. Student *t*-test were used to calculate the difference between each groups and significance was assed at the $p<0.05$ and experiments were performed independently at least twice.

Results

Effects of GZ on pancreatitis signs

Administration of cerulein resulted in elevation of lipase and amylase activities in serum of mice, but when we treated the mice with GZ, we found that GZ reduces both amylase and lipase activities in a dose dependent manner (**Figure 1A** and **1B**). Twelve hours after induction of AP, pancreases were subjected to determination of water content for evaluation of pancreas edema. As shown in **Figure 2**, edema was robustly increased in the AP group. However, GZ administration significantly reduced it to the baseline level. Histological examination of pancreas sections 12 h after cerulean injections showed tissue damage characterized by infiltration of inflammatory cells and necrosis of acinar cells. As shown in **Figure 3** the severity and extent of histological signs of pancreas injury were significantly decreased in GZ-treated AP mice (**Figure 3**).

GZ decreases serum levels of MCP-1 and MIP-2 in experimental mice

MCP-1 and MIP-2 have been shown to play crucial roles in migration of inflammatory cells particularly monocytes and neutrophils [20, 21]. When we examined the levels of these two cytokines in the serum of the AP group, we observed that the levels of MCP-1 and MIP-2 significantly elevated. However, treatment of cerulean-induced AP with GZ decreased the serum levels of both MCP-1 and MIP-2 in a dose dependent-manner (**Figure 4**).

Effect of GZ on infiltration of inflammatory cells into pancreas

We have previously reported a method, by employing flow cytometry analysis, for enumerating leukocytes infiltration in the pancreas tissue [19]. In general, our flow cytometry data show that isolated pancreatic cells from all four groups of mice consisted of a wide variety of cells as evidenced by side scatter (FSC) and forward scatter (SSC) (**Figure 5A**). Next, we identified each population of infiltrating inflammatory cells in pancreas tissue by employing CD45/SSC gating. As shown in **Figure 5B**, all three

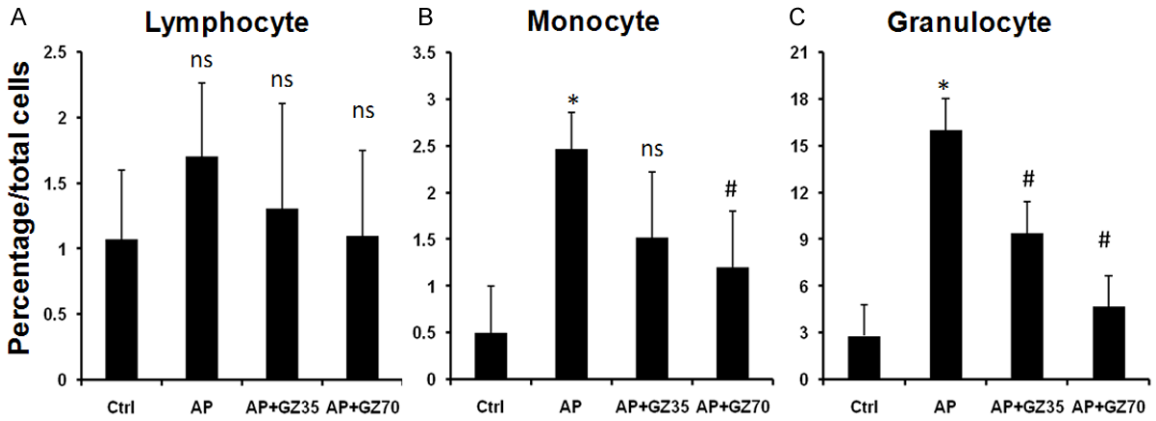

Figure 6. Quantifying the percentage of all inflammatory cells by flow cytometry. Inflammatory cells were determined as stated in **Figure 5B** and the percentage of each cell population was analyzed by dividing the number of each population to total of isolated pancreatic cells. The percentage of lymphocytes, monocytes and granulocytes are shown in graphs A, B and C, respectively. Pooled data from 6-8 mice per group are shown. Values are the mean percentage of each population ± SD. *P<0.05; ns: non-significant. **P*<0.05 compared with the control mice; #*P*<0.05 compared with the AP group; ns; non-significant.

inflammatory cells including granulocytes, monocytes and lymphocytes were present in the pancreas of normal, cerulein-induced AP and GZ-treated AP mice. However, the number of these inflammatory cells was quite different in the four experimental groups. Finally, the percentage of each granulocytes, monocytes and lymphocytes was analyzed by dividing the number of each population to the total number of isolated pancreatic cells. We found that the percentage of both monocytes and granulocytes were robustly increased in the AP than the control group. However, when we administrated GZ to the mice with AP, we found that GZ reduced the percentage of monocytes and granulocytes in pancreas in a dose-dependent manner (**Figure 6B** and **6C**). In contrast, we observed that not only the number of lymphocytes did not significantly enhance in the AP group, but also GZ did not show any significant effect on the number of these cells (**Figure 6A**).

Discussion

Since various inflammatory cells and cytokines have been shown to play many crucial roles in pathophysiology of AP, this disease has been regarded as an inflammatory disease [1, 22]. Therefore, finding new pharmaceutical agents targeting the signaling pathways of inflammatory mechanisms in AP are of great interest. In the current study, we examined the anti-inflammatory effects of GZ on experimental AP and found that GZ significantly reduces the histopathological signs of AP as well as reducing the enzymatic activity of amylase and lipase. Moreover, GZ treatment not only decrease the serum level of MCP-1 and MIP-2 in the serum of cerulein-induced AP mice, but also remarkably reduced the number of infiltrated granulocytes and monocytes in the pancreas tissue.

We utilized cerulein-induced experimental AP as a typical animal model of AP which is enzymologically and histologically resembles to AP in human [23]. Our data demonstrate that GZ treatment led to reducing of both serum lipase and amylase activities as well as histopathological features of AP implying that GZ have beneficial effects on experimental AP. Even though our work is the first study showing the beneficial effects of GZ on AP in animal model there are many reports demonstrating that GZ have either protective or therapeutic effects on many inflammatory diseases [15, 16, 24]. Ram A et al have recently shown that GZ markedly reduced infiltration of eosinophils in peribronchial area and inhibited ovalbumin-induced airway constriction in experimental allergic asthma in mice [16].

MCP-1 has been shown to play in important role in migrating and trafficking of monocytes [20]. In addition, MCP-1 is also a key player in the pathogenesis of AP, and previous studies have clearly shown that inhibition of MCP-1 by either a dominant-negative mutant MCP-1 gene or its inhibitor reduces the histopathological

features of AP [25, 26]. More importantly, clinical studies have revealed that serum levels of MCP-1 is elevated in patients with AP [4]. In the current study, we demonstrated that the serum levels of MCP-1 is significantly increased in the cerulean-induced AP confirming the previous study [27]. However, Gz administration in mice with AP led in a significant reduction of serum MCP-1 levels. Accordingly, previous *in vitro* studies has clearly shown that GZ reduces MCP-1 production by human monocytes [28] and granulocytes [29]. In addition, pancreatic acinar cells have shown to be one of the major sources of MCP-1 secretion in AP [30]. We have to consider that GZ might directly affect on these MCP-1-producing cells which need to be further elucidated.

Mounting evidence demonstrated that neutrophils migrate and accumulate into pancreas tissue during AP leading to activating of many proenzymes and secreting of various inflammatory mediators [9, 31]. MIP-2 have been shown, through binding to its receptor CXCR2, to act as strong chemoattractant for neutrophils in mice [32]. Migration of neutrophils into lung was significantly reduced in LPS-induced acute lung injury in $CXCR^{-/-}$ mice [33] indicating that MIP-2 plays a crucial role in trafficking of neutrophil into the pancreas in AP. Herein, we demonstrate that the levels of MIP-2 is significantly increased in the serum of mice with AP. In contrast, when we treated the mice with GZ the levels of MIP-2 remarkably decreased. In line with our data, a recent study showed that GZ reduced IL-4 and IL-5 in the BAL of experimental allergic mice [16] indicating that GZ may inhibit production of inflammatory cytokines.

Neutrophil excessive in pancreas has been contributed to the severity of AP, and deletion of these inflammatory cells resulted in attenuating of AP in animal models [10]. We have recently employed flow cytometry to enumerate inflammatory cells in the pancreas tissue and found it as a reliable approach for quantifying these cells [19]. In the current study, we observed that the number of lymphocytes did not increase in the AP group and GZ administration did not show any effect on the number of these cells in the pancreas tissue. In contrast, the numbers of monocytes were significantly enhanced in the AP mice and GZ treatment decreased their number. Accordingly, as MCP-1 is the main chemoattractant factor for monocytes migration [20], we found that the amount of MCP-1 is reduced in the serum of GZ-treated AP mice.

Many studies have shown that neutrophils contribute to pathobiology of many inflammatory diseases such as AP [10, 34], lung injury [33, 35] and arthritis [36]. Further supporting of the notion is our previously study exhibiting that the percentage of infiltrated neutrophil consist of 18.2 of total pancreatic cells in the AP mice, whereas it was only 2.9 in the control mice [19]. Herein, we observed that GZ administration remarkably decreased the number of infiltrated neutrophils in the pancreas tissue of AP mice in a dose-dependent manner. This is in accordance with our ELISA data revealing that GZ treatment reduces the levels of MIP2, a major chemoattractant factor for neutrophil, in the serum of AP mice.

So far, nutritional support is the only available treatment for AP and many experimental and clinical studies indicate that enteral nutrition reduces length of hospital stay and mortality in patients with AP [37]. Licorice and its derivatives such as GZ and glabridin possess anti-inflammatory effect in many experimental animal models [17, 24, 29]. Base on data, we postulate that adding GZ to enteral nutrition might reduce have some beneficial effects in patients with AP which needs further investigations.

Taken together, we demonstrate for the first time that GZ administration attenuates AP in mice and reduces infiltration of leukocytes particularly neutrophils in the pancreas tissue. Thus, GZ might have a potential to inhibit inflammatory responses in patients with AP.

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Disclosure of conflict of interest

None.

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